

MULTIDRUG RESISTANCE PROTEIN 4 (MRP4/ABCC4) EXPRESSION IS REGULATED BY HISTAMINE IN ACUTE MYELOID LEUKEMIA CELLS, DETERMINING cAMP EFFLUX

Angela Rodríguez González¹, Ana Sahores², Antonela Díaz-Nebreda¹, Agustín Yaneff², Nicolás Di Siervi², Natalia Gómez², Federico Monczor², Natalia Fernández², Carlos Davio², and Carina Shayo¹.

¹Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires, Argentina.

²Instituto de Investigaciones Farmacológicas (ININFA-UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina.

Corresponding author

Carina Shayo PhD. Instituto de Biología y Medicina Experimental (IBYME-CONICET). Vuelta de Obligado 2490 (1428). Buenos Aires, Argentina. Tel: +54-11-4783-2869 ext. 1241. carinashayo@hotmail.com, cshayo@dna.uba.ar

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Running title

Histamine H₂ receptor and MRP4 as targets for AML.

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Abbreviations

AML, acute myeloid leukemia; AUC, area under curve; BSA, bovine serum albumin; dbcAMP, dibutyryl cAMP; e-cAMP, extracellular cAMP; Epac, Exchange protein activated by cAMP; FAB, French-American-British classification; FBS, fetal bovine serum; FRET, Fluorescence Resonance Energy Transfer; GPCR, G protein coupled receptor; h, hour; i-cAMP, intracellular cAMP; IL-2, interleukin-2; min, minute; MRP, multidrug resistance-associated protein; PBS, phosphate-buffered saline; PDE, phosphodiesterase; PKI, Protein kinase inhibitor-(14-22)-amide, myristoylated; qPCR, quantitative RT-PCR; RBP, radio binding protein assay; ROS, reactive oxygen species; RPMI, RPMI 1640; s, second.

Abstract

Intracellular cAMP (i-cAMP) levels play an important role in acute myeloid leukemia (AML) cell proliferation and differentiation. Its levels are the result of cAMP production, degradation, and exclusion. We have previously described histamine H₂ receptors and MRP4/ABCC4 as two potential targets for AML therapy. Acting through histamine H₂ receptors histamine increases cAMP production/synthesis, while MRP4/ABCC4 is responsible for the exclusion of this cyclic nucleotide. In this study, we show that histamine treatment induces MRP4/ABCC4 expression, augmenting cAMP efflux and that histamine, in combination with MRP inhibitors, is able to reduce AML cell proliferation. Histamine, through histamine H₂ receptor, increases i-cAMP levels and induces MRP4 transcript and protein levels in U937, KG1a, and HL-60 cells. Moreover, histamine induces MRP4 promoter activity in HEK293T cells transfected with histamine H₂ receptor (HEK293T-H₂R). Our results support that the cAMP/Epac-PKA pathway, and not MEK/ERK nor PI3K/AKT signaling cascades, is involved in histamine mediated upregulation of MRP4 levels. Finally, the addition of histamine potentiates the inhibition of U937, KG1a, and HL-60 cell proliferation induced by MRP4 inhibitors. Our data highlight that the use of a poly-pharmacological approach aimed at different molecular targets would be beneficial in AML treatment.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells, which arises in the bone marrow. These cells fail to differentiate, do not respond to normal regulators of cell proliferation, and neither undergo programmed cell death. Although current chemotherapeutic drugs are highly toxic and frequently poorly tolerated by AML patients, chemotherapy is the treatment of choice for the induction therapy and one of the options for post-remission therapy in this

pathology. Almost all patients who do not receive post-remission treatment eventually relapse [1–3]. Histamine, acting via histamine H₂ receptors on AML blasts and phagocytes, inhibits the production and release of oxygen free radicals, protecting cytotoxic T lymphocytes and natural killer cells [4]. In this way, interleukin-2 (IL-2) activates cytotoxic cells more efficiently, which results in the elimination of tumor cells. Based on this mechanism, treatment with histamine and IL-2 in post-remission therapy was found to significantly reduce relapse in AML patients [5–7].

Alternatively, another potentially less toxic approach in AML treatment is the use of drugs which induce cell differentiation. This kind of therapy is based on the assumption that many neoplastic cell types exhibit reversible differentiation defects, which, upon appropriate treatment, results in tumor reprogramming and induction of terminal differentiation. To date, this has been successful in acute promyelocytic leukemia therapy [8–10] and recently with enasidenib in mutant isocitrate dehydrogenase-2 (IDH2) relapsed or refractory AML [11,12].

Many reports underline the role of cAMP in blood cell proliferation, differentiation and apoptosis in normal and leukemic cell population [13–16]. Dibutyryl cAMP (dbcAMP) and cAMP increasing agents induce monocytic differentiation in both mouse and human leukemic cells [17,18]. The importance of time-course cAMP signaling to achieve leukemic cell differentiation has also been described [19]. In this sense, despite the fact that activating histamine H₂ receptor causes a rise in cAMP levels, rapid receptor desensitization prevents cell differentiation in U937 cells [20–22].

Classically, the regulation of cAMP signaling involves, a) cAMP production through a Gs protein-coupled receptor (GsPCR), b) cyclic nucleotide degradation by phosphodiesterases (PDEs), and c) GPCR desensitization. Recently, a novel mechanism of intracellular cAMP (i-cAMP) regulation was described in several systems, which involves cAMP efflux through multidrug resistance-associated protein (MRPs) transporters. Among them, MRP4/ABCC4, MRP5/ABCC5, and MRP8/ABCC11 were specifically associated to cGMP and cAMP efflux out of the cells, in addition to other substrates [23–26]. Likewise, MRP4 transcript and protein expression were found to be upregulated by cAMP in HeLa cells, vascular smooth muscle cells, megaloblastic M70e cells, and pancreatic adenocarcinoma cell lines [27,28]. Moreover, we previously demonstrated that MRP4 has a relevant role in the regulation of i-cAMP levels in AML cell lines, determining cell proliferation and differentiation [29].

Thus, the genetic or pharmacological negative modulation of MRP4 in AML cells in culture and in an *in vivo* AML xenograft model caused the inhibition of cell proliferation and the induction of cell differentiation and apoptosis [29,30].

Taking in consideration the aforementioned, the purpose of the present study was to evaluate the ability of histamine to regulate MRP4 levels in AML cells, its impact on cAMP efflux and, in turn, whether histamine potentiates the antiproliferative effect of MRP4 inhibitors. These studies will provide new insights in the management of leukemia therapies, involving histamine H₂ receptors and MRP4 as combined therapeutic targets.

Results

Histamine increases MRP4 expression through histamine H₂ receptor in AML cells.

In order to evaluate the role of histamine in the modulation of MRP4 expression in AML cell lines, MRP4 mRNA transcript levels were assessed by qPCR in U937 cells, a leukemia promonocytic model (FAB M5), after 24 h exposure to 100 μ M histamine. Following cell exposure, histamine led to an approximate 1.5-fold increase in MRP4 transcript levels as compared with untreated cells. In the same way, treatment with amthamine, a specific histamine H₂ receptor agonist, increased MRP4 mRNA levels, while TFMP-HA or VUF8430, histamine H₁ and H₄ receptor agonists respectively, did not modify MRP4 transcript levels (Fig. 1A, left). The effect of histamine H₃ receptor agonists was not assayed as AML cells do not express histamine H₃ receptors. As histamine H₂ receptor is coupled to Gs protein, thus enhancing cAMP production, we assayed the capacity of dbcAMP, a classical cell membrane-permeable cAMP analog, to raise MRP4 transcript levels. As expected, after 24 h exposure, 0.4 mM dbcAMP increases mRNA MRP4 levels in U937 cells (Fig. 1A, left). Also, rolipram, a PDE4-selective inhibitor, showed a synergistic effect with histamine on MRP4 mRNA levels (Fig. 1A, right). The effect of histamine was specific for MRP4 transporter, since after 24 h exposure, MRP5 and MRP8 transcript levels were not modified (data not shown). In accordance, MRP4 protein levels, determined by western blot, significantly increased when U937 cells were exposed for 72 h to histamine and amthamine, but were not modified upon treatment with histamine H₁ or H₄ receptor agonists (Fig. 1B). Other leukemic cell lines, KG1a (FAB M0/M1) and HL-60 (FAB M2), were subjected to the same experimental approach. In both cell lines, treatment with

histamine and amthamine also increased MRP4 protein levels, indicating that MRP4 induction by histamine H₂ receptor activation is not restricted to U937 cells only (Fig. 1C).

Next, we evaluated the effect of histamine H₂ receptor overexpression on MRP4 levels. In U937 cells stably transfected with histamine H₂ receptor (U937-H₂R), higher basal mRNA and protein MRP4 levels were observed compared to U937 parental cells (Fig. 2A and B). Likewise, treatment with 10 μ M amthamine, as well as with 25 μ M forskolin (an adenylyl cyclase activator), augmented MRP4 mRNA levels in this clone (Fig. 2A). We then evaluated the effect of treatment with three histamine H₂ receptor inverse agonists (cimetidine, ranitidine and famotidine) on transcript and protein MRP4 levels. Upon treatment with 10 μ M of either histamine H₂ receptor inverse agonists, U937-H₂R cells showed a marked decrease in mRNA and protein MRP4 levels (Fig. 2C and D). Since there is no strong evidence about the presence of endogenous histamine in U937 cells [31,32], we pointed to the constitutive activity of the histamine H₂ receptor [33,34] as responsible for MRP4 upregulation.

To further associate the regulation of MRP4 levels with the capacity of the ligands to modulate the intracellular cAMP (i-cAMP) levels, we first evaluated the i-cAMP levels in U937 and U937-H₂R cells in a basal state and after a 30 min stimulation with 10 μ M amthamine. As expected, i-cAMP basal levels are higher in U937-H₂R cells compared to parental cells and increase upon stimulation of histamine H₂ receptor in both systems. Likewise, treatment with 10 μ M histamine H₂ receptor inverse agonists decreases basal i-cAMP levels, while 25 μ M forskolin increases them in U937-H₂R cells (Fig. 2E).

Altogether, these findings show that histamine, acting through the histamine H₂ receptor, increases i-cAMP levels and upregulates MRP4 levels in AML cell lines.

Mechanisms of MRP4 regulation by histamine H₂ receptor.

We then addressed MRP4 promoter activity by luciferase reporter assays in HEK293T cells transfected with histamine H₂ receptor (HEK293T-H₂R). In this system, both histamine and amthamine were able to induce MRP4 promoter activity by approximately 70% respect to basal activity (Fig. 3A). This suggests that MRP4 transcription stimulation, and not a higher stability in mRNA transcripts, is responsible for MRP4 mRNA and protein upregulation. Moreover, FRET assays using Epac-S^{H187} as a cAMP molecular sensor confirmed the induction of i-cAMP levels caused by histamine and amthamine treatment in HEK293T-Epac-S^{H187}-H₂R cells (Fig. 3A, right).

Given that histamine H₂ receptors act as potent stimulators of i-cAMP accumulation and activate PKA in various native cells and in cells expressing recombinant histamine H₂ receptors [34,35], we next addressed whether this kinase is responsible of mediating histamine and amthamine-induced MRP4 upregulation. Interestingly, the widespread used PKA inhibitor, H89, failed to inhibit both histamine and amthamine-mediated induction of MRP4 expression (Fig. 3B). Nevertheless, western blot analysis confirmed that the inhibitor prevents CREB phosphorylation (pCREB), a PKA substrate, denoting H89 inhibits PKA activity effectively (Fig. 3B right). We also assessed whether the activation of Epac (Exchange protein activated by cAMP) is involved in the histamine and amthamine induction of MRP4 promoter activity. For this, we cotransfected HEK293T cells with a dominant negative mutant of Epac (N-Epac), which has been shown to abolish endogenous Epac activity by competition. In the presence of this construct, the histamine and amthamine response was abrogated (Fig. 3B). Given that Epac proteins behave as specific GTP exchange factors for the Ras GTPase family members of Rap, we further evaluated Rap1 downstream participation in Epac-mediated histamine and amthamine response. The Rap1GAP construct, which accelerates Rap1 GTP hydrolysis, prevented histamine and amthamine-induction of MRP4 promoter (Fig. 3B).

It has been previously described that histamine and amthamine are able to modulate ERK and AKT activity, positively and negatively, respectively, in an independent manner of PKA and Epac activity [36,37]. We observed that PD98059, a specific MEK inhibitor, failed to modulate the histamine and amthamine response upon MRP4 promoter (Fig. 3C, left), although it effectively prevented histamine and amthamine activation of ERK (pERK), as determined by western blot (Fig. 3C, right). Next, to clarify whether the decrease in AKT activity caused by histamine or amthamine treatment is involved in the induction of MRP4 promoter activity, the constitutive active form of AKT (AKT-Myr) was expressed in HEK293T-H₂R cells. In this way, even in the presence of AKT active protein (pAKT), confirmed by western blot (Fig. 3D, right), we observed that treatment with histamine and amthamine still increases MRP4 promoter activity (Fig. 3D, left).

To elucidate whether these signaling pathways are implicated in histamine-mediated induction of MRP4 transcript levels in AML cells, U937 cells were treated with Epac, PKA and MEK inhibitors. The MEK inhibitor PD98059, did not significantly modify the histamine-induced MRP4 levels, evaluated by qPCR (Fig. 4). Conversely, the Epac inhibitor ESI-09 and the PKA inhibitors H89,

KT5720, and PKI, a more selective PKA inhibitor, prevented MRP4 upregulation by treatment with histamine (Fig. 4).

Collectively, these results support that the cAMP/Epac-PKA pathway, and not MEK/ERK nor PI3K/AKT signaling cascades, is involved in histamine mediated upregulation of MRP4.

Histamine regulates cAMP efflux in AML cells.

Given that histamine treatment increases the transcript and protein levels of MRP4 in AML cells through cAMP signaling, and that cAMP, in turn, is a MRP4 substrate, we further evaluated whether long-term histamine treatment is able to increase cAMP efflux. For this, we measured intracellular and extracellular cAMP (e-cAMP) levels and estimated cAMP exclusion process in U937 non-treated cells and in U937 cells incubated with histamine for 48 h (Fig. 5 left). As an indicator of cAMP efflux, we plotted the % of cAMP exclusion, and observed a significant increase upon long-term treatment with histamine in U937 cells (Fig. 5 right). Interestingly, this higher capacity of exclusion in histamine-treated U937 cells is inhibited by ceefourin-1, a novel MRP4 specific inhibitor [38] (Fig. 5).

Altogether, these results indicate that the higher MRP4 expression levels induced by long-term treatment with histamine cause a greater cAMP efflux that can be reversed by MRP4 pharmacologic inhibition.

Histamine enhances the antiproliferative effect of MRP inhibitors on AML cells.

Considering that, in AML cells, histamine treatment upregulates MRP4 levels through the cAMP/Epac-PKA pathway causing an increase in cAMP efflux and, in this way, prevents cAMP accumulation in the intracellular compartment, which is necessary to inhibit cell proliferation, we analyzed the effect of combining histamine treatment with MRP4 inhibitors for 72 h upon U937 cell proliferation. Since previous work has shown that probenecid (a general inhibitor of transmembrane organic anion transporters) impairs U937 cell proliferation enhanced by amthamine treatment [29], we assessed U937 cell proliferation and viability in cells exposed to probenecid, MK571 (a MRP1, 2, and 4 inhibitor), and ceefourin-1, at different concentrations. All three agents reduced cell proliferation and only affected cell viability at the highest tested concentration (Fig. 6A). Notably, the addition of histamine potentiates the antiproliferative effect of the three MRP4 inhibitors in U937, KG1a, and HL-60 cells (Fig. 6B).

Discussion

In the present study, we evaluated the effect of histamine in the regulation of MRP4 levels in AML cells, together with its repercussion on cAMP efflux and its relationship with AML cell proliferation. We found that: 1) MRP4 is transcriptionally upregulated upon treatment with histamine, specifically through histamine H₂ receptor; 2) cAMP/Epac-PKA pathway is involved in this modulation; 3) as a consequence of MRP4 increased expression by histamine, cAMP efflux is concomitantly augmented; and 4) histamine enhances the antiproliferative effect of MRP4 inhibitors in AML cells.

Histamine achieves diverse functions in the organism, both physiological and pathological, through the activation of four GPCR (histamine H₁-H₄ receptors) [34,35,39]. From the pharmacological point of view, numerous ligands that antagonize the effects of histamine H₁ and H₂ receptors have been developed for decades with the purpose of alleviating allergic symptoms and controlling acid gastric secretion, respectively [39–41]. Recently, the use of histamine was approved for the treatment of AML in the post-remission stage, acting as an histamine H₂ receptor agonist, and became the first approved therapeutic agent to target immunosuppression in AML [5,42]. According to our results, the administration of histamine to AML patients would produce undesirable effects in the remaining leukemic cells as it would increase MRP4 levels, which definitely would not be beneficial for the subsequent treatment and/or progression of the illness. Leukemia therapy involves the use of nucleoside analogs such as cytarabine, 6-mercaptopurine, 6-thioguanine, both in the induction and post-remission stages of this disease. It was reported that MRP4 facilitates the efflux of these analogs [43–45]. In this way, an increase in MRP4 protein levels would prevent the accumulation of these drugs and their active derivatives, which could lead to therapeutic failure. Likewise, an increase in MRP4 levels could also influence the efficacy of other nucleoside analogs used in AML treatment, such as clofarabine or fludarabine [45–47]. On the other hand, comparison of different AML cell subtypes showed a positive correlation between MRP4 expression levels and the degree of aggressiveness [48,49]. Thus, the highest levels of MRP4 were found in the least differentiated cell subtypes, including leukemia stem cells.

It is also important to highlight that according to a pharmacokinetic study performed in healthy individuals who received the daily doses of histamine approved for AML therapy, the histamine plasma levels were around the order of nanomolar [50]. In addition, MRP4 upregulation by treatment

with histamine was detected in *in vitro* assays with leukemia cell lines. Therefore, future approaches are necessary to understand the real impact of these molecular mechanisms described in the development and treatment of the disease in patients.

Histamine, through the activation of histamine H₂ receptor, regulates several signaling pathways. In addition to increasing cAMP levels by activating this Gs coupled GPCR, histamine inhibits PI3K/Akt/mTOR and stimulates Ras/MEK/ERK pathways [36,37]. Likewise, it was described that histamine decreases intracellular free radical levels in monocytic leukemia cells [51]. In both assayed systems, HEK239T cells transfected with the histamine H₂ receptor and U937 leukemic cells, histamine upregulated MRP4 levels through the activation of the cAMP pathway. Our results are in agreement with what was previously observed in human megakaryoblastic leukemia M07e cells, vascular smooth muscle cells, HeLa and PDAC cells, where cAMP increases MRP4 levels [27,28].

However, we detected differences between the cAMP effectors involved in MRP4 regulation in HEK293-H₂R cells and U937 cells, describing the participation of Epac or Epac and PKA, respectively. Certainly, the differences in cellular environments including stoichiometry and compartmentalization of proteins make it possible to have different readouts of cAMP signaling. Although we have not assessed downstream signaling events in leukemic cells, it is possible that PKA may converge with Epac in Rap1 activation. The activation of Rap1 by PKA was recently described as a key signaling node for follicular thyroid carcinogenesis [52].

Likewise, in this study, we found that the regulation of MRP4 exerted by histamine is independent of the MEK/ERK pathway unlike MRP4 modulation by cAMP in the aforementioned systems. Since reactive oxygen species (ROS) have been associated with the upregulation of multidrug transporters in several systems [53–55], high levels of ROS present in leukemic cells [56,57] could be playing a crucial role in basal MRP4 expression levels. However, even if treatment with histamine decreases the levels of intracellular ROS in monocytic AML cells [51], the concomitant increase in i-cAMP levels would have a fundamental role in the upregulation of MRP4 levels.

The positive modulation of MRP4 levels induced through the activation of histamine H₂ receptor as a mechanism to control i-cAMP levels is not the only regulatory mechanism described in AML cells. *Delgado and collaborators* have demonstrated that histamine stimulation of histamine H₂ receptor induces PDE activity in U937 cells, suggesting PDE protein synthesis [58]. Moreover, the induction

of the PDE activity has been described in response to a prolonged stimulation of GsPCRs and to the overexpression of histamineH₂ receptor in U937 cells [59,60].

On the other hand, the rapid desensitization of histamine H₂ receptor in U937 cells avoids high sustained i-cAMP levels and, therefore, prevents histamine treatment from triggering AML cell differentiation [19]. In this cell line, the inhibition of GRK2, the enzyme responsible for histamine H₂ receptor's desensitization, allowed cells to differentiate upon histamine treatment [22]. Altogether, the fast desensitization of histamine H₂ receptor, the increase in PDEs activity, and the upregulation in MRP4 expression described in this work, are responsible for the antiproliferative control evasion mechanism, triggered by i-cAMP in AML cells.

The increase in MRP4 levels induced by the prolonged treatment with histamine resulted in an increase in the ability of the cell to exclude cAMP. It is important to note that the exclusion assays were carried out in the absence of PDEs inhibitors. Thus, we can conclude that cAMP exclusion mechanism plays a fundamental role in the regulation of i-cAMP levels, even in the presence of active PDEs. This has already been observed in other systems such as cardiac myocytes and sperm cells, where MRP4 controls cAMP homeostasis through the efflux of this nucleotide, crucial for cardiac development and sperm capacitation [61,62]. In addition to modulating i-cAMP levels, MRP4 upregulation also affects e-cAMP levels. It would be of interest to evaluate the possible role of e-cAMP in the proliferation of leukemic cells, given that it has been recently described in PDAC cells that e-cAMP plays a proliferative role through a still unknown receptor [63].

Diverse studies show that, in addition to MRP4, MRP5 and MRP8 induce the extrusion of cAMP, although with lower affinity [26]. Regarding AML cells, we have previously shown that MRP4 is a key participant in cAMP transport [29]. We have determined that cAMP efflux in U937 shMRP4 cells is comparable to that of U937 cells treated with probenecid, which inhibits general transport activity. Moreover, we observed that cAMP exclusion levels of U937 shMRP4 cells remain unaltered upon treatment with probenecid. These findings indicated that MRP5 and MRP8 do not have a crucial role in cAMP efflux in this cell line.

Previously, we reported that amthamine, through the increase of cAMP levels, potentiates the antiproliferative effect of probenecid, a general inhibitor of transporters, in U937 cells, as it allows cell differentiation *in vitro* [29]. On the other hand, recent studies report that histamine may exert a

direct antileukemic impact, performing pro-differentiating effects on human monocytic NOX2+ AML cells *in vitro* and *in vivo* [51]. In this work, we demonstrate that histamine potentiates the inhibition of AML cell proliferation caused by probenecid and by other more specific MRP4 inhibitors, such as MK571 and ceefourin-1. Likewise, ceefourin-1 proved to exert an antiproliferative effect in astrocytes as a consequence of cAMP efflux inhibition [64]. When considering the implementation of preclinical *in vivo* tests to evaluate the combined effect of histamine and a MRP4 inhibitor, it would be interesting to evaluate ceefourin-1 effect *in vivo*, as no reports indicate its use in animal models yet. Regarding probenecid, the FDA has approved its clinical use in chronic gout and gouty arthritis treatment and has shown significant antiproliferative and apoptotic effects in *in vivo* AML xenografts [30]. Future studies in *in vivo* models will indicate the possibility of the implementation of probenecid in a drug-repositioning scheme in combination with histamine.

We conclude that in AML cells, histamine treatment augments MRP4 levels, with the consequent increase in cAMP efflux, and that the simultaneous treatment with an MRP4 inhibitor enhances its antiproliferative capacity.

It is known that tumor cells have different strategies/mechanisms to compensate or evade adverse situations. In this sense, a poly-pharmacological approach directed to different molecular targets would be beneficial in cancer treatment. Our study indicates that histamine H₂ receptor and MRP4 transporter are two very interesting membranous proteins, which could be susceptible to modulation in AML treatment not only to achieve the apoptosis/differentiation of leukemic cells, but also to boost the patient's immune response against the tumor and to improve the sensitivity of tumor cells to nucleoside based chemotherapeutic agents, preventing development of resistance.

Materials and Methods

Materials. Cell culture medium, antibiotics, bovine serum albumin (BSA), cAMP, dibutyl cAMP (dbcAMP), rolipram, forskolin, 2,3-trifluoromethylphenylhistaminedimaleate (TFMP-HA), VUF8430 dihydrobromide, H89 and ESI-09 were obtained from Sigma-Aldrich. Amthamine dihydrobromide, histamine dihydrochloride, PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), MK571 3-[[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid, KT5720, Protein kinase inhibitor-(14-22)-amide,

myristoylated (PKI) and ceefourin-1 5-[(2-Benzothiazolylthio)methyl]-2,4-dihydro-4-methyl-3*H*-1,2,4-triazole-3-thione were acquired from Tocris Cookson Inc. (Ballwin, MO, USA). Fetal bovine serum (FBS) was purchased from Natocor (Córdoba, Argentina). Other chemicals used were of analytical grade and obtained from standard sources.

Plasmid constructions. pCEFL-HA-H₂R and pGL3-MRP4-Luc were previously generated in our laboratory [21,28]. pCMV-Myc-N-Epac and pMT2-HA-Rap1GAP plasmids were kindly provided by Dr. Omar Coso (Department of Physiology and Molecular Biology, FCEN, UBA, Buenos Aires, Argentina). pACL4-AKT1-Δ4-129 (AKT-Myr) was a gift from Dr. Virginia Novaro (Laboratorio de Carcinogénesis Hormonal, IBYME-CONICET, Buenos Aires, Argentina). The mTurquoise2-EPAC-cp173Venus-Venus (Epac-S^{H187}) construct was provided by Dr. Kees Jalink (Cell Biophysics & Imaging Group, Netherlands Cancer Institute, Netherlands) [65].

Cell culture and transfections. HEK293T cell line and human leukemia cell lines (U937, KG1a and HL-60) were obtained from the American Type Culture Collection (ATCC) and grown in a humidified 5% CO₂ atmosphere at 37 °C in DMEM medium and RPMI 1640 (RPMI) medium respectively, supplemented with 10% FBS and 50 µg/ml gentamicin. The U937-H₂R clone was generated in the laboratory by stable transfection. Briefly, U937 cells were harvested by centrifugation from cultures in exponential growth phase, washed in phosphate-buffered saline (PBS), and resuspended at 2 x 10⁷ cells/ml in fresh RPMI medium on ice. pCEFL-HA-H₂R (10 µg) linearized with SalI was added to the cell suspension (400µl) and kept on ice for 5 min. Cells and DNA were then subjected to a pulse of 150V at a capacitance of 250 µF using a Gene Pulser (Bio-Rad, Hercules, CA, USA). Cells were returned to ice for 5 min and incubated in anon-selective medium overnight. Cells were then plated in a 96-well culture plate in RPMI medium containing 0.8 mg/ml G418. After 2-3 weeks, the surviving clones were amplified and checked for histamine H₂ receptor expression by real-time PCR (data not shown). Mock clones were generated upon the stable transfection with the PCEFL empty vector. cAMP and MRP4 levels were similar to those of parental U937 cells.

The clone HEKT Epac-S^{H187} was generated by stable transfection. HEK293T cells (5 x 10⁵/well) were seeded in a 24-well plate 24 h before transfection and cDNA construct was transfected using the K2 Transfection System (Biontexas, Munich, Germany), as indicated by the supplier. Clonal selection was carried out for two weeks in the presence of 25 µg/ml zeocine (InvivoGen) in 96-well plates. Then,

clones were tested in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) by fluorescence spectra (450-650 nm) after excitation at 430 nm. The clone with the highest emission was select for the assays, and was maintained in DMEM with 10% FBS, 50 µg/ml gentamicin and 12.5 µg/ml zeocin. For transient transfection, HEK293T or HEKT Epac-S^{H187} cells were grown to 80-90% confluency and the cDNA constructs were transfected using the K2 Transfection System. The transfection protocol was optimized as recommended by the supplier (Biontex). Unless indicated, all assays were performed 48 h after transfection.

RT-PCR and Quantitative Real-time PCR. Total RNA was isolated from U937, KG1a and HL-60 cells using Quick-Zol reagent (Kalium Technologies, Buenos Aires, Argentina) following the manufacturer's instructions. For the first-strand cDNA synthesis, 2µg of total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (AB) with random primers. Quantitative real-time PCR (qPCR) was performed in triplicate using the resulting cDNA, the HOT FIREPol Eva Green qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) for product detection, and the following primers: human MRP4 forward, 5'-GGACAAAGACAACCTGGTGTGCC-3' and reverse, 5'-AATGGTTAGCACGGTGCAGTGG-3'; and human β-actin forward, 5'-GGACTTCGAGCAAGAGATGG-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3'. The cDNA was amplified by 45 cycles of denaturing (30 s at 95 °C), annealing (30 s at 60 °C), and extension (30 s at 72 °C) steps. The specificity of each primer set was monitored by analyzing the dissociation curve, and the relative mRNA quantification was performed using the comparative ΔΔCt method using β-actin as the housekeeping gene. Triplicate samples of at least three independent experiments were analyzed.

Western blot assay. Cells were lysed in 50 mM Tris-HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue and sonicated to shear DNA. Nitrocellulose membranes were cut to detect MRP4 and α-tubulin proteins in the same blot. The top part was revealed with anti-MRP4 antibody (M4I-10, Thermo Scientific, Waltham, MA, USA) and the bottom part with anti α-tubulin (Abcam, Cambridge, UK), as a loading control. Blots for pCREB/α-tubulin and pERK/ERK-1 were re-probed after stripping. pCREB, pERK, ERK-1 and pAKT antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). We previously established that all antibodies used in this study revealed a specific band in full-length

western blots. Anti-rat (Santa Cruz Biotechnology), anti-mouse or anti-rabbit (Vector Labs, Burlingame, CA, USA) antibodies conjugated to HRP were used and developed with the ECL Western Blot Detection Reagent (Amersham Biosciences, Little Chalfont, UK). Bands were quantified through Image J analysis software [66].

Reporter gene assay. HEK293T cells at 80% confluence seeded on 24-well plates were cotransfected with the pGL3-MRP4-Luciferase reporter plasmid and pCEFL-HA-H₂R vector. In some experiments, cells were cotransfected with the plasmid constructs indicated in the corresponding figure legend or an empty vector to maintain the total amount of DNA. After 6 h, cells were seeded in 96-well plates for 24 h and were deprived from FBS for another 16 h. Cells were then stimulated with the corresponding agents and luciferase activity was measured 24 h later with the Steady-Glo Luciferase assay system, according to the manufacturer's instructions (Promega Biosciences Inc. San Luis Obispo, CA, USA) using the GloMax 96 Microplate Luminometer (Promega Biosciences Inc.). Experimental reporter activity was normalized to control activity. Triplicate samples of at least three independent experiments were analyzed.

cAMP radiobinding protein (RBP) assay. Cells were exposed for different periods with different concentration of the different agents, centrifuged, and 1 ml of ethanol was added to the supernatants and pellets. The alcohol was then evaporated to concentrate extracellular cAMP (e-cAMP) and intracellular cAMP (i-cAMP) respectively, and resuspended in 50 mM Tris-HCl, pH 7.4, 0.1% BSA for cAMP determination. Cyclic AMP content was determined by competition of [³H]cAMP for PKA, as described previously [67]. Triplicate samples of three independent experiments were tested.

FRET time-course of cAMP intracellular levels. HEK293T cells stably transfected with Epac-S^{H187} were plated in 96-well plate and transiently transfected with pCEFL-HA-H₂R.

After washing twice with NaCl 0.9%, the plate was placed in a FlexStation 3 at 37 °C and the fluorescence signal after excitation at 430 nm was recorded at 475 nm (donor) and 530 nm (FRET), as a measure of i-cAMP basal level. After the addition of histamine or amthamine, the donor and FRET signals were recorded every 20 s during 480 s and the ratio FRET/donor was normalized to basal levels (R/R₀). An AUC value of 480 s R/R₀ i-cAMP response was calculated for each assay. Triplicate samples of three independent assays were tested.

Assessment of cell viability. To determine cell viability using the trypan blue exclusion assay, we diluted an aliquot of cell suspension with the same volume of 0.4% trypan blue solution and incubated it for 5 min at room temperature. The percentage of viable cells was quantified using a hemocytometer chamber. Triplicate samples of three independent experiments were tested.

Proliferation assay. Cells growing in exponential phase were exposed for 72 h to MRP4 inhibitors at different concentrations with or without histamine (100 μ M), as indicated in the corresponding figure legend, and the number of cells was quantified in a hemocytometer chamber. The assays were carried out by triplicate in at least three independent experiments.

Statistical analysis. Analyses of the statistical significance from assays were performed using GraphPad Prism 6.00 Software (San Diego, CA). Data are shown as the mean \pm SD of at least three independent experiments (n=3), each performed in triplicate. Group comparisons were assessed by using one- or two-way ANOVA, followed by Dunnett's or Bonferroni's as the post hoc test. Tukey's multiple comparison tests were used for comparisons of four or more groups. Statistical significant differences were calculated by two-tailed unpaired t-test. Values of $P < 0.05$ were considered to indicate statistically significant differences. In all cases, minimum sample size to ensure an adequate statistical power (.0.95) was determined with G*Power 3.1 software [68,69] using standard deviation values of emerging experimental data as input.

Author Contributions

FM, NF, CD and CS conceived the study, ARG, NG, CD and CS designed the experiments, ARG, AS, ADN, NDS and AY performed the experiments, ARG, ADN, NDS, AY, NG, CD and CS analyzed data; NF, AS and CS wrote the manuscript; ARG, ADN, AY, NDS, NG, FM and CD revised the manuscript. CD and CS supervised the study; AY, FM, NF, CD and CS acquired the funding.

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Conflicts of Interest

The authors have no conflict of interest.

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Figure legends

Figure 1: Effect of histamine on MRP4 levels in AML cells. A. U937 cells were exposed to 100 μ M histamine (HIST), 10 μ M TFMP-HA (H_1 R ago), 10 μ M amthamine (H_2 R ago), 10 μ M VUF8430 (H_4 R ago), 0.4 mM dbcAMP, 10 μ M rolipram (ROLI) or 10 μ M rolipram+100 μ M histamine for 24 h and MRP4 mRNA levels were quantified by qPCR. Results were normalized to β -actin mRNA levels and expressed relative to control (left) or to 100 μ M histamine (right). B. U937 cells were exposed to 100 μ M histamine (HIST), 10 μ M TFMP-HA (H_1 R ago), 10 μ M amthamine (H_2 R ago), or 10 μ M VUF8430 (H_4 R ago) for 72 h and MRP4 and α -tubulin protein levels were quantified by western blot. C. KG1a and HL-60 cells were exposed to 100 μ M histamine (HIST) or 10 μ M amthamine (H_2 R ago) for 72 h and MRP4 and α -tubulin protein levels were quantified by western blot. B-C. Left, densitometric quantification of MRP4 protein bands normalized to α -tubulin and expressed relative to control. Right, representative western blot assay. Results are shown as mean \pm SD (n=3). One-way ANOVA followed by Dunnett's post hoc test. ns: not significant, *P<0.05; **P<0.01; ***P<0.001 respect to control.

Figure 2: Effect of histamine H_2 receptor activity on MRP4 levels in U937 cells. A-C. U937 or U937- H_2 R cells were exposed to 10 μ M amthamine (H_2 R ago), 25 μ M forskolin (FK), 10 μ M cimetidine (CIM), 10 μ M ranitidine (RAN), or 10 μ M famotidine (FAM) for 24 h and MRP4 mRNA levels were quantified by qPCR. Results were normalized to β -actin mRNA levels and expressed relative to control. B-D. U937 or U937- H_2 R cells were exposed to 10 μ M cimetidine (CIM), 10 μ M ranitidine (RAN) or 10 μ M famotidine (FAM) for 72 h and MRP4 and α -tubulin protein levels were quantified

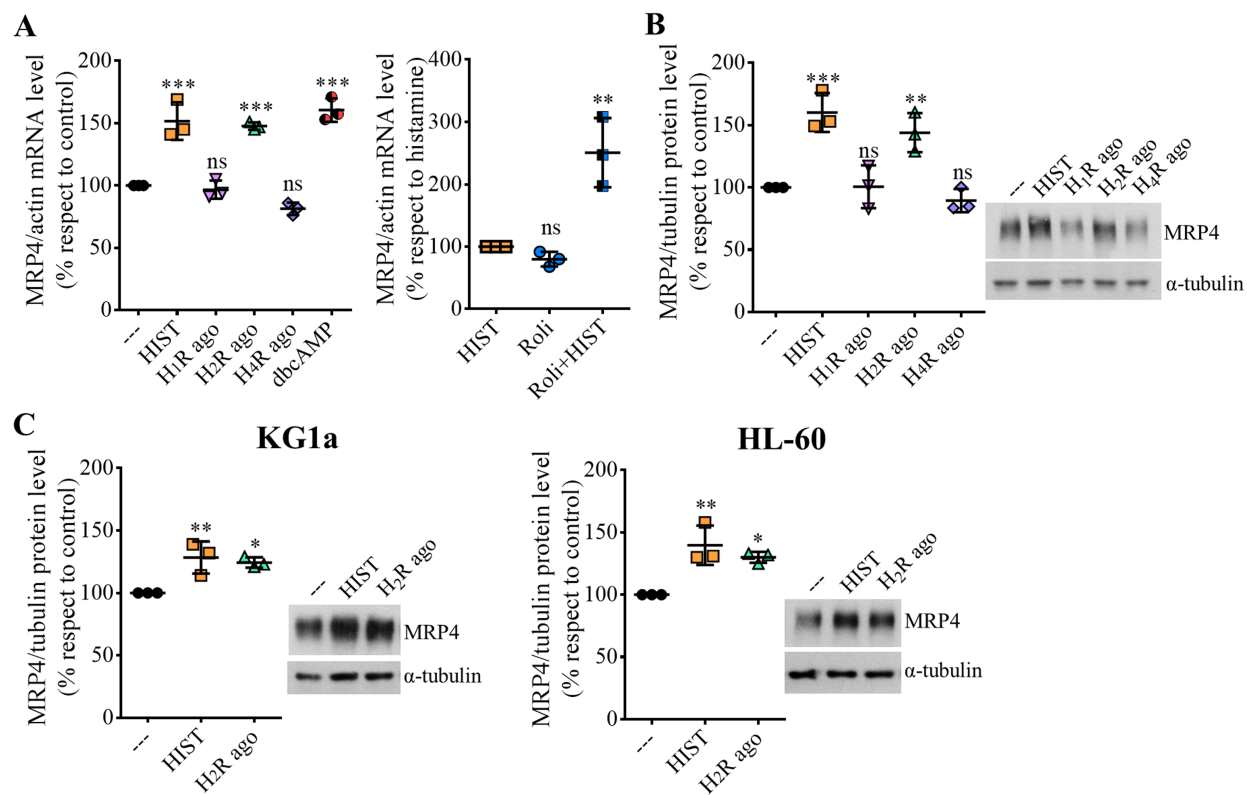
by western blot. Left, densitometric quantification of MRP4 protein bands normalized to α -tubulin and expressed relative to control. Right, representative western blot assay. E. U937 or U937-H₂R cells were stimulated during 30 min with 10 μ M amthamine (H₂R agonist), 10 μ M cimetidine (CIM), 10 μ M ranitidine (RAN), 10 μ M famotidine (FAM) or 25 μ M forskolin (FK) and i-cAMP levels were determined as detailed in Materials and Methods. Results are shown as mean \pm SD (n=3). Two-tailed unpaired t-test, one-way ANOVA followed by Bonferroni or Dunnett post hoc test. *P<0.05; **P<0.01; ***P<0.001 respect to U937 control cells. ##P<0.01; ###P<0.001 respect to U937-H₂R control cells.

Figure 3: Effect of different pathways modulated by histamine through histamine H₂ receptor on MRP4 promoter activity in HEK293T-H₂R cells. HEK293T cells were cotransfected with histamine H₂ receptor, MRP4-Luc, and MOCK, N-Epac, or Rap1GAP. After two days, cells pretreated or not with 10 μ M H89 or 50 μ M PD98059 (PD) were stimulated with 100 μ M histamine (HIST) or 10 μ M amthamine (H₂R ago). Luciferase activity was measured as indicated in Materials and Methods and all results are shown as mean \pm SD (n=3 or 5). One-way ANOVA followed by Dunnett's post hoc test, two-way ANOVA followed by Tukey's multiple comparison test. A. ***P<0.001; **P<0.01 respect to control. B, C-D. 100 % corresponds to pretreated or transfected cells without histamine or amthamine treatment. ***P<0.001 respect to histamine treatment in control cells, ### P<0.001 respect to amthamine treatment in control cells. A right. Effect of 100 μ M histamine (HIST) or 10 μ M amthamine (H₂R ago) on i-cAMP levels. AUC values of 8 min-time course FRET changes in histamine H₂ receptor-transfected HEK293T Epac-S^{H187} cells measured in a FlexStation 3 after the addition of the corresponding stimulus. B-C right. HEK293T cells transfected with histamine H₂ receptor were pretreated or not with 10 μ M H89 or 50 μ M PD98059 for 15 min and stimulated with 100 μ M histamine (HIST) or 10 μ M amthamine (H₂R ago) for 5 min. pCREB, pERK, ERK and α -tubulin protein levels were quantified by western blot. Representative western blot assay (n=3). D right. HEK293T cells transfected with histamine H₂ receptor and AKT-Myr construct were treated with 10 μ M amthamine (H₂R ago) for 5 min. pAKT protein levels was quantified by western blot. Representative western blot assay (n=3).

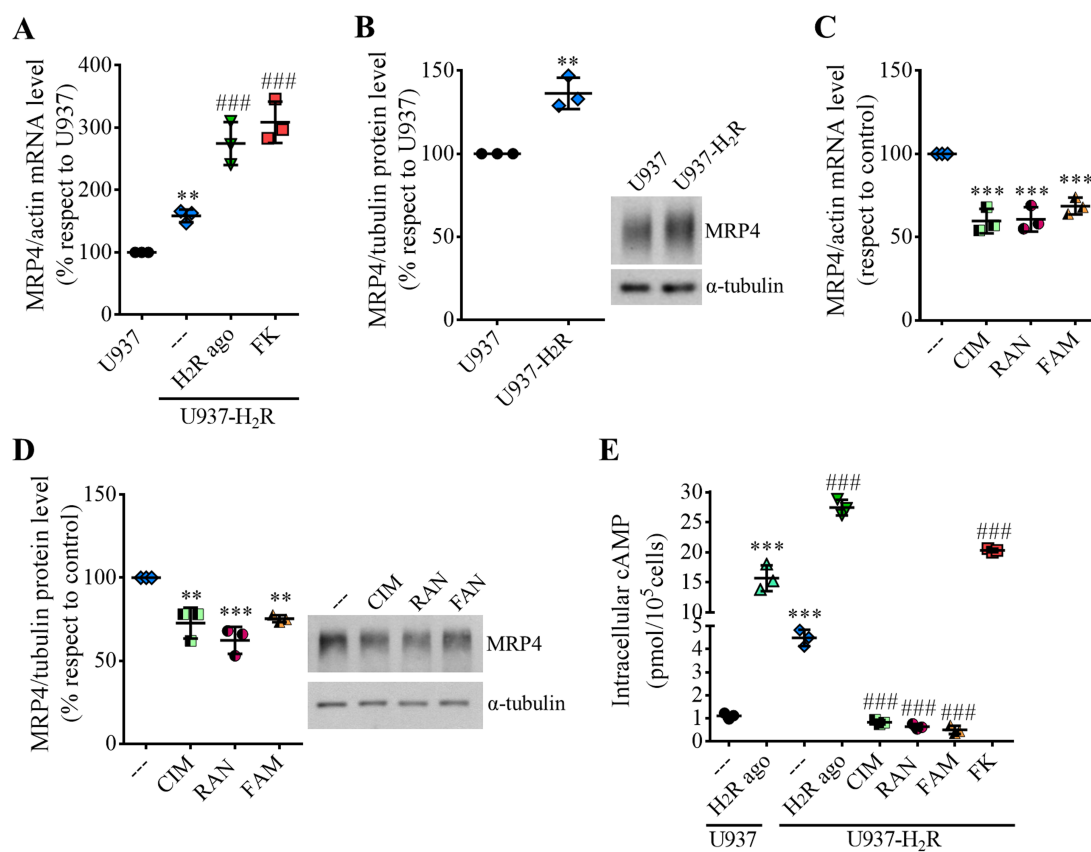
Figure 4: Effect of PKA, Epac and MEK/ERK activity on histamine-induced MRP4 mRNA levels in U937 cells. U937 cells pretreated with 10 μ M H89, 10 μ M KT5720, 10 μ M PKI, 5 μ M ESI-09, or 50 μ M PD98059 (PD) for 15 min were exposed to 100 μ M histamine (HIST) for 24 h and MRP4 mRNA levels were quantified by qPCR. Results were normalized to β -actin mRNA levels. 100 % corresponds to pretreated cells without histamine treatment. Results are shown as mean \pm SD (n=3). One-way ANOVA followed by Dunnett's post hoc test. ***P<0.001 respect to histamine stimulation in cells without pretreatment.

Figure 5: Effect of histamine treatment on cAMP efflux in U937 cells. U937 cells were treated with 100 μ M histamine for 48 h, washed, and basal i-cAMP and e-cAMP levels were measured by RBP after 120 min incubation in fresh culture media in the presence or not of 25 μ M ceefourin-1. Left: raw data, Right: % of cAMP exclusion calculated as: $e\text{-cAMP} \times 100 / (i\text{-cAMP} + e\text{-cAMP})$. Data is shown as mean \pm SD (n=3). Two-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001.

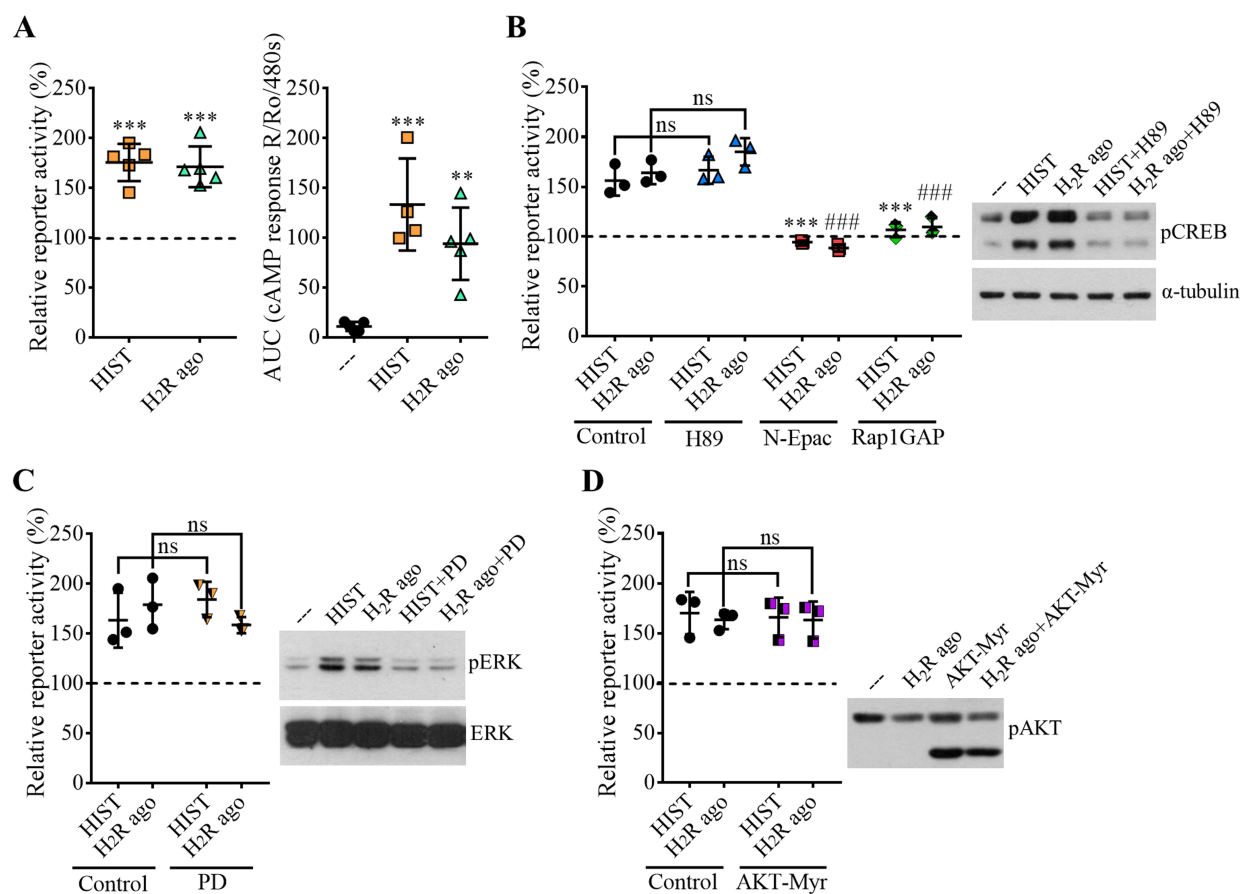
Figure 6: Effect of treatment with histamine and MRP4 inhibitors on AML cell proliferation. A. U937 cell proliferation and viability in the presence of different concentrations of probenecid, MK571 and ceefourin-1 for 72 h were determined by cell count. B. U937, KG1a and HL-60 cell proliferation in the presence of 100 μ M histamine and different concentrations of probenecid, MK571, or ceefourin-1 for 72 h was determined by cell count. 100% corresponds to non-treated cells. Results are shown as mean \pm SD (n=3). Two-way ANOVA followed by Tukey's multiple comparison test. ns: not significant, *P<0.05, **P<0.01; ***P<0.001.



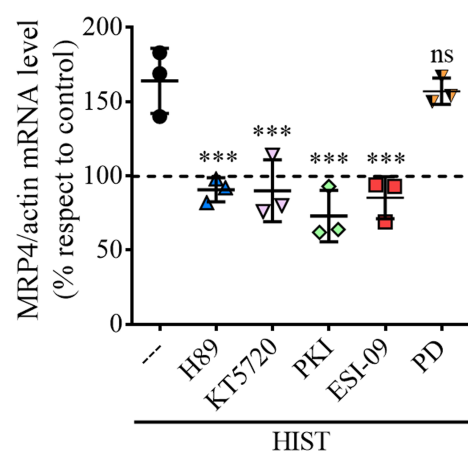
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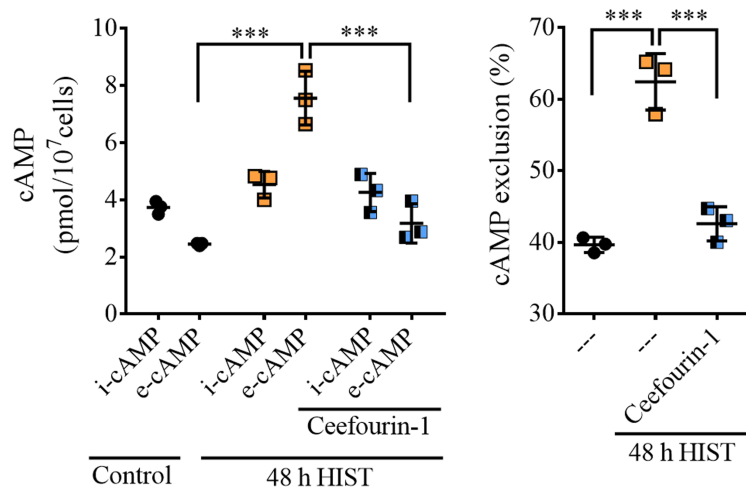
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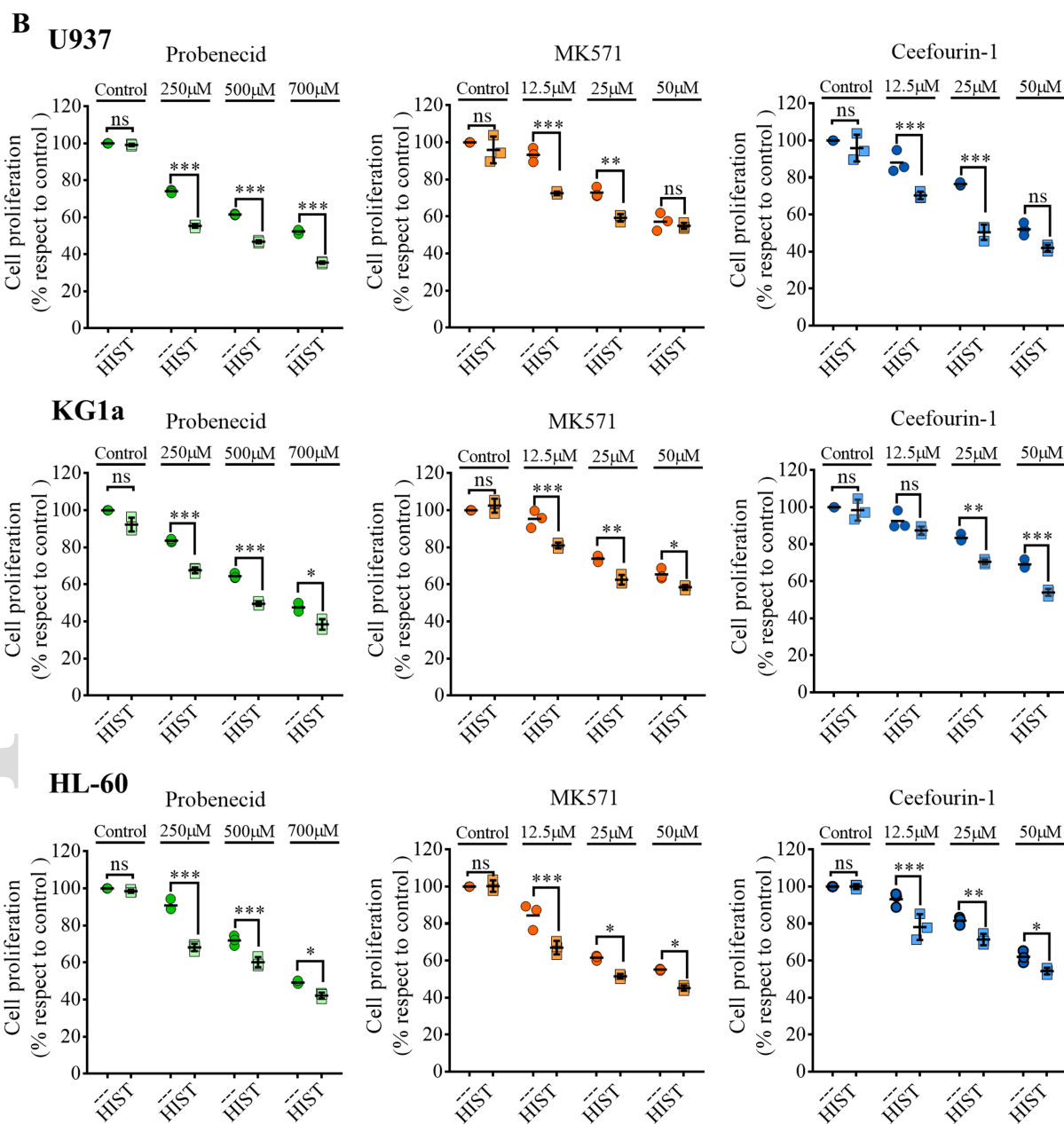
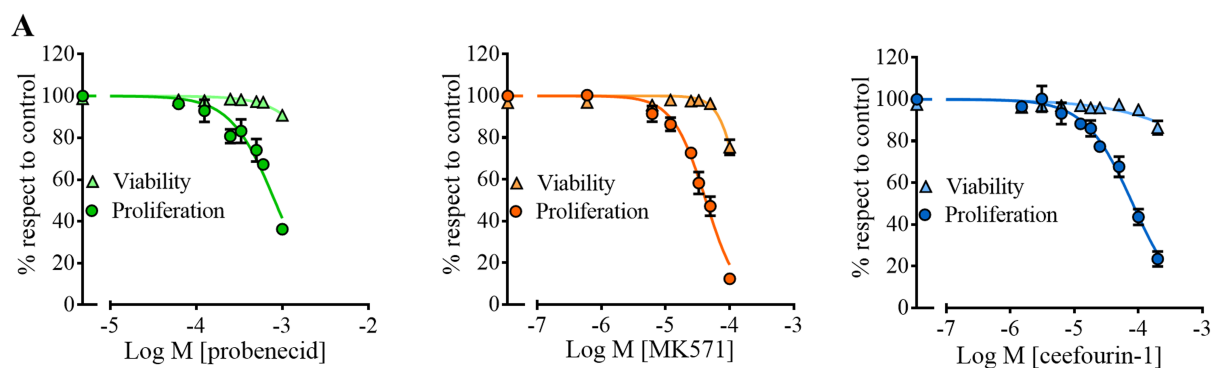
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